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(54) Title: STRAIN OF BACTERIA OF THE SPECIES *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI*, COMPOSITION THEREOF FOR USE IN FOOD AND PRODUCT CONTAINING SAID STRAIN

(57) Abstract

Strain of *Lactobacillus* useful as probiotics in food and naturopathic medicines and which is resistant *in vitro* against hydrochloric acid and gastric juices and tolerates bile salts without deconjugating them whereas strong assimilation is occurring and which has good survival at the passage through the stomach and the gastrointestinal tract and which strain is growing optimally at about 37 °C, which strain is *Lactobacillus paracasei* subsp. *paracasei*, which is a Gram-positive, homofermentative, rod-shaped bacterium capable of producing L-lactic acid and containing three plasmids having a size of 2.2, 4.36 and 9.1 Kb, respectively. The invention also relates to a composition containing the strain and a product consisting of or containing a concentrate of the strain.

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STRAIN OF BACTERIA OF THE SPECIES *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI*, COMPOSITION THEREOF FOR USE IN FOOD AND PRODUCT CONTAINING SAID STRAIN

The present invention relates to a strain of Lactobacillus paracasei subsp. paracasei, a composition thereof for use in food as well as a product containing said strain.

Definition and Characterisation of the Strain

The novel strain (which in the following for simplicity will be designated LMG P-17806) is a variant of the species Lactobacillus paracasei subsp. paracasei. It has the characteristics of the species with a GC-content of 44%. LMG P-17806 has been isolated from samples from the gastrointestinal micro-flora of humans. LMG-P-17806 is a Gram-positive, homofermentative rod-shaped bacteria. It produces L-lactic acid (laevorotatory stereoisomer of lactic acid) and grows optimally at 37°C. The strain is characterised by being tolerant in-vitro against hydrochloric acid and gastric juice by tolerating bile salts without deconjugating them and by having a great ability of assimilating cholesterol. The strain is also characterised by containing three plasmids having a size of 2.2, 4.36 and 9.1 Kb respectively. Other characteristics are that the strain is fermenting ribose, adonitol, galactose, glucose, fructose, mannose, sorbose, mannitol, sorbitol, N-acetyl-glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, D-turanose and D-tagatose. On the other hand it does not ferment glycerol, erythritol, D- and L-arabinose, D- and L-xylose, β-methyl-D-xyloside, rhamnose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, arbutin, salicin, melibiose, raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose D- and L-fucose, D- and L-arabitol and 2- and 5-ketogluconate.

The strain has been characterised by SDS gel electrophoresis, in which it has been compared to six other strains of Lactobacillus paracasei subsp. paracasei, vide the accompanying figure. In this comparison it has been shown to differ from all

other described strains and at the same time as it when being compared to other lactobacilli appears to belong to the designated species. It has also been characterised with regard to ribosomic RNA in a so called Riboprinter®. With this method the strain has been shown to possess 76% similarity with the type strain for Lactobacillus paracasei subsp. paracasei and 72% similarity to the type strain of Lactobacillus casei analysed at the same occasion.

The strain has been deposited at Belgian Coordinated Collections of Microorganisms - BCCM, LMG collection, and there been given the accession No. LMG P-17806.

The Advantages of the Strain

LMG P-17806 has, when compared to known strains of Lactobacillus, crucial advantages in the use as probiotics in food and naturopathic medicines by a unique combination of good properties;

- the strain has good resistance against gastric juice and bile salts, but unlike many other strains it does not deconjugate the bile salts;

- it has a great ability to assimilate cholesterol;

- the strain is well managing the passage through the stomach;

- the strain has an influence on the conditions in the model of large intestine by increasing the production of L-lactic acid therein;

- the strain is not more pro-inflammatory than common yoghurt bacteria;

- the strain prevents intestinal cells from being invaded by pathogenic microorganisms, such as Salmonella typhmurium;

- the strain has an antagonistic action against the gastric ulcer bacterium Helicobacter pylori:

- the strain forms bacteriocins which are active against clostridiae;

- the strain survives well in milk as well as in frozen and dried form;

- the strain, unlike most other lactobacilli has a favourable influence on the taste of fermented milk products (does not give any tang).

The present strain of Lactobacillus paracasei subsp. paracasei can be used as an additive to food or as naturopathic medicine, so called "Medical Food", or as an additive to naturopathic medicine.

Such medicines can be used for children with the purpose of alleviating atopic problems; for elderly persons in order to correct altered microflora caused by normal alterations by age or an altered secretion of hydrochloric acid; and for persons in general in order to normalise the intestine flora, whereas the content of clostridium bacteria is decreasing, lactobacilli and bufido bacteria being increased and high contents of coliformic bacteria being decreased.

By means of these properties the strain LMG P-17806 differs from previously known strains, which will be shown in the examples below.

Preparation of the Strain

The strain is prepared in the usual way for lactobacilli. A substrate suited for lactobacilli is used. This substrate should for instance contain at least one of the carbohydrates which the strain can ferment according to what is stated above, in combination with proteins, vitamins, minerals and other nutrients which normally are required by lactobacilli. Examples of suitable commercial substrates are yeast extract-glucose broth, MRS (de Man-Rogosa-Sharp broth), Rogosa, milk added with a minor amount of a yeast extract, etc. The strain is cultivated microaerophilicly or in the complete absence of

oxygene, suitably at a temperature between +15°C and +42°C. If the substrate is grafted with 0.1 to 1 % of graft a culture time of between 10 and 40 hours is suitable. The strain can, if desired, be concentrated by centrifugation or filtration whereafter the concentrate is washed in order to remove the culture medium. The concentrate can then be frozen or lyophilized in the common way. In this way preparations of between 100 millions and 100,000 millions of living bacteria LMG P-17805 per g can be prepared. A preparation can then be used as such or be used as an additive to food, for instance to milk or another product which gives LMG P-17806 the possibility to survive and, if desired, to grow.

INVESTIGATIONS

A. Investigation Concerning the Passage of the Strain Through the Gastrointestinal Tract

LMG P-1706 was cultivated in the way described above and added together with yoghurt culture to milk. A fermented product was produced by incubating the milk for five hours at +42°C. A palatable product was obtained which contained fully 100 millions living LMG P-17806 per gram of product. Healthy persons were given 3 x 200 g product daily for one weak. The total intake of LMG P-17806 was between 40 billions and 200 billions.

Faeces samples were examined before the intake, after one week of consumption and one week after the intake had ceased. As is evident from Tables 1 and 2 below, a strong increase in the number of lactobacilli in the test subjects was obtained. Two isolates per test subject were classified as to species on each occasion, i.e. 20 isolates in total. 18 of the isolates for the consumption time appeared to consist of LMG P-17806 according to fenotypical classification. This bacteria strain was not discovered in the samples before or after the intake of LMG P-17806.

In average the contents of the faeces samples during the supply were very high and varied only moderately from 63 millions to

320 millions per gram, i.e. with a factor of 5. Noteworthy was, that the contents were largely the same independant of what contents were measured before the start of the experiment. After the supply had ceased the contents reverted to what seems to be natural for the test subject in question.

In Tables 1 and 2 below the content of lactobacilli in faeces was determined, in millions per gram, by plating and using the substrate Rogosa.

Table 1

5 test subjects with originally low content of lactobacilli

Test subject	Before the experiment	During the experiment	After the experiment
1	0.05	120	0.07
2	0.15	320	0.12
3	0.08	97	0.14
4	0.009	63	0.02
5	<0.001	278	0.002

Table 2

5 test subjects with high contents of lactobacilli

Test subject	Before the experiment	During the experiment	After the experiment
6	1.2	297	1.3
7	0.7	83	0.6
8	0.2	136	0.18
9	4.3	74	3.5
10	0.6	212	0.8

The examination shows that the strain has good survival at the passage through the gastrointestinal tract.

B. Examination Concerning the Formation of L-lactic Acid by the Strain in a Model of Large Intestine

The fermented product above was added to a so called SHIME-reactor which is an in vitro model of the intestine. Samples were taken from the part of the reactor which corresponds to the most important parts of the large intestine. Similar comparative tests were carried out with some other Lacto bacillus strains, i.a. closely related L. paracasei subsp. paracasei. As is evident from the following Table 3 below LMG P-17806 gave a strong increase in the production of L-lactic acid, which is the very lactic acid isomer which is generated by LMG P-17806. A production of lactic acid is considered as favourable for several reasons, i.a. considering the anti-bacterial effect of the lactic acid as well as the fact that a lower pH is supposed to reduce the availability and formation of nitrogen compounds.

Table 3

Production of L- and D-lactic acid in a SHIME-reactor after the addition of different lactic acid bacteria in mg per litre reactor content

Lactic acid bacteria	Reactor 4		Reactor 5		Reactor 6	
	L	D	L	D	L	D
LMG P-17806	300	80	500	100	280	90
L. paracasei	40	80	200	70	130	40
L. rhamnosus	90	10	10	70	80	10
L. plantarum	60	50	60	70	40	50

In the table "L" refers to the laevorotatory isomer of lactic

acid and "D" to the dextrorotatory isomer.

Reactor 4 corresponds to the upper part of the large intestine, reactor 5 to the middle part and reactor 6 to the lower part of the large intestine. The investigation shows that the strain is forming L-lactic acid in the model of the large intestine.

C. Investigation of How the Strain is Protecting Intestinal Epithelium Cells from Invasion of *Salmonella Typhimurium*

Intestinal epithelium cells of the type CaCo-2 cells were cultivated in-vitro. These were added with a combination of lactobacilli and Salmonella typhimurium in the ratio 100:1 with the addition of 1 million salmonella per ml. The effect was studied after incubation for 120 min at 37°C. The amount of invading salmonella was determined by washing the plates with adhering CaCo-2 cells three times. The adhering cells were treated with the antibioticum gentamycin in a concentration of 100 mg/l for one hour in order to kill all bacteria which had not invaded cells. Then the plates were washed with PBS in order to remove all gentamycin and finally the entrapped bacteria were released by treating the CaCo-2 cells with 0.1 % Triton-X during shaking. The number of salmonella was then determined by common plating methodology. LMG P-17806 had a pronounced effect in that it reduced a number of invaded cells. The closely related paracasei-variant 506 on the other hand seemed rather to stimulate the invasion of salmonella bacteria. Also with regard to this property LMG P-17806 showed a positive effect. The results are reported in Table 4 below.

Table 4

Invasion of salmonella

Lactic acid bacteria	% invading salmonella	
	without lactic acid bacteria	with lactic acid bacteria
LMG P-17806	2.5	0.75
L. paracaseiK 506	2.5	7
L. plantarium	2	2
L. rhamnosus	2.5	0.75

The table shows that the strain LMG P-17806 gives a marked protection against invasion of salmonella bacteria.

D. Investigation of Protection Against *Helicobacter Pylori*

In a mouse model where the mice had been infected with Helicobacter pylori the effect of supplying a fermented milk product with a strain of LMG P-17806 on one hand and without said strain on the other on the content of H. pylori measured in faeces was examined.

The mice were infected with 100 millions of the strain H. Pylori 17874 in helical form at three occasions with an interval of one day. Then the mice were given experimental products and the content of H. pylori in faeces was measured by means of heparinised magnetic balls and Enzyme Immuno Assay. Three products were examined. All the fermented milk products appeared to reduce the share of H. pylori, but the effect was occurring considerably faster in the cases when the product contained LMG P-17806 in comparison to common yoghurt and in comparison with a strain of L. fermentum KLD, respectively.

Table 5

Content of H. pylori in faeces measured by heparinised magnetic balls and Enzyme Immuno Assay

Product	before intake	after intake for 2 days	after intake for 7 days	7 days after cease of intake
Common yoghurt	1.48	1.86	0.63	2.25
Yoghurt with L. Fermentum	1.65	1.62	0.94	1.61
Yoghurt with LMG P-17806	1.80	0.68	0.61	1.71

The figures in the table state the absorbency of 405 nm and are relative contents.

E. Examination of the Influence of the Strain LMG P-17806 on the Immunological Defence

The immunological defence system is controlled by a series of signal substances, so called cytokins. Some of these can be proinflammatory. The influence of LMG P-17806 on the production of cytokins TNF-alfa and IL-6 was compared with the influence of the two species contained in a yoghurt culture, L. delbruckei subsp. bulgaricus and Streptococcus thermophilus. leucocytes were separated from human blood and added to living bacteria or bacteria killed with glutaraldehyde in an amount of 10 millions of leucocytes. As control lipopolysacharides (LPS) from E.-coli were used. The results are reported in Table 6 below. The results show that the LMG P-17806 has the same inflammatory properties as a common yoghurt culture in the model used.

Table 6

The influence of the strain LMG P-17806 on the immunological defence

Lactic acid bacteria	TNF-alpha mg/l		IL-6 mg/l	
	living	killed	living	killed
S.thermophilus,E584	12	1	0.4	<0.1
L. bulgaricus, E585	2	3	0.5	0.1
LMG P-17806	4	1	0.7	<0.1

F. Examination of the Resistance of the Strain LMG P-17806 Against Antibiotics

Probiotics can be useful for use in connection with disorders in the balance of the intestine flora during medication with antibiotics. At the same time it is important that probiotics do not contribute to spreading of resistance to antibiotics, and this is especially important in the contemporary use of probiotics and antibiotics. The resistance of the strain LMG P-17806 against different antibiotics has been established for that reason. The sensitivity of the strain LMG P-17806 to different antibiotics were determined by establishing the content at which a reduction in the growth of the strain by 50 %, measured as optical density, was obtained.

It appeared that the strain LMG P-17806 was resistant against vancomycin and was not inhibited even by 256 mg/l. The strain showed some resistance against trimethoprim and cefotaxime, an optical density (OD) of 50% at 12 and 4 mg/l, respectively being obtained. The strain LMG P-17806 was on the other hand sensitive to chloraphenicol, erythromcyin, rifampicin and tetracycline, where already levels below 1 mg/l resulted in an inhibition of the growth by 50%.

The influence by antibiotics was also examined as a survey by using so called Sensi discs from Oxoid. According to these results LMG P-17806 was resistant against aztreonam, ceftaximid, cefoxitin, colistine sulphate, kanamycin, polymyxin B, streptomycin and vancomycin.

LMG P-17806 might thus according to these results be especially interesting for use at the same time as therapy with antibiotics such as vancomycin, trimethoprim as well as several cefloxacins, which all are antibiotics with known side effects on the intestine flora and intestine function.

THE USE OF THE STRAIN LMG P-17806

Example 1 Preparation of Bacterial Concentrate

A frozen bacterial concentrate with 10 billions of LMG P-17806 per gram was prepared in a the way stated above by cultivating the lactobacilli in a substrate of whey added with 1 g yeast extract per litre at a constant pH of 5.5 for 14 hours at 36°C. The bacteria were separated by centrifugation with continuos washing of the centrifugate. The concentrate was frozen in liquid nitrogen and then stored at -80°C until use.

Example 2 Preparation of Fermented Milk Product

A product milk was prepared from milk by homogenising the milk, heat-treating it at +95°C for five minutes and tempering it to +37°C. The product milk was grafted with 0.01 % of a commercial, frozen yoghurt culture and 0.5 % of the LMG P-17806 concentrate. The cultures were allowed to grow for six hours at the temperature stated. The milk had then coagulated and the pH decreased to 4.55. The coagulated form was broken and the product chilled to +12°C whereafter it was packed in common plastic cups, which are normally used for yoghurt, and after-cooled in a refrigerating chamber having a temperature of +5°C for one day and night. pH had then decreased to 4.4. The product was then stored at +8°C for up to three weeks.

The content of the strain LMG P-17806 was monitored and as is seen from the results in Table 7 below, there was a limited growth of the strain LMG P-17806 survived storing for three weeks at pH below 4.4 very well.

Table 7

The storability of the strain LMG P-17806 in milk product

Graft	Content in product milk	Content after cultivation	After storing for 1 week	After storing for 2 weeks	After storing for 3 weeks
9900	49	97	132	118	121

The contents are given in millions per gram.

The product had a normal appearance with a separation of whey of barely 1 % after storing for fourteen days. The product tasted excellently and had uniform consistency and a fresh, mild flavour. The test product received better judgements than normal yoghurt in an independent consumer survey carried out by an research institute. The product was compared in a consumer survey, in which the testing persons did not know what product was tasted, with a corresponding product without the strain LMG P-17806. The product with LMG P-17806 was preferred by 74% of the persons of the testing panel, and it received the average value of 7.6 in a scale of 9 points, which is significantly higher than the result for common yoghurt. The judgement was slightly more than 1 point higher than a previously known probiotic culture with Lactobacillus acidophilus. The results also differ from those previously obtained in comparison between pure yoghurt and mixed products of yoghurt and the probiotic bacteria Lactobacillus. acidophilus and Bifidobacterium longum, respectively. No significant differences could be noted between pure yoghurt

and the respective mixed product in these comparisons.

Example 3 Suitable Addition of the Strain LMG P-17806

Experiments were also carried out in order to find out the suitable range for the addition of the strain LMG P-17806. Milk was treated and then added with yoghurt culture according to the above, whereas the addition of concentrate of the strain LMG P-17806 was varied from 5% to 0.01%. At such a high content as 5% graft of LMG P-17806 a tang was obtained, which probably originated from components of the graft itself. The change in pH was normal, however, and the appearance of the product was normal. The content of LMG P-17806 in fresh product was 504 millions per gram and the contents remained at this level during the storage. At the addition of 0.01% LMG P-17806 concentrate the product could not sensoricly be distinguished from common yoghurt and the content of LMG P-17806 was already after one week less than one million per ml, which is the lowest content a product must contain in order to be allowed to state the product to contain a specific probiotic according to proposal to international legalislation.

Example 4 Preparation on Fermented Special Product

The experiment was carried out as above except that the milk also was added with 0.4 g yeast extract per litre. The graft was performed with 0.01% of the same yoghurt culture as above, but only 0.1% LMG P-17806 concentrate was added. Incubation was carried out at +34°C for 8 hours whereafter the process was broken off and the product chilled, packed and stored as in Example 3 above. LMG P-17806 grew ten times under these conditions and similarly to the above the bacteria survived well during storage. The results are reported in the Table below.

Table 8

The storability of the specific milk product of the strain LMG P-17806

Graft	Content in milk product	Content after cultivation	After storing for one week	After storing for two weeks	After storing for three weeks
9900	10	103	114	109	107

The contents in the table are given in millions per gram product.

The product had a normal appearance with a separation of whey of 1.5% after storing for fourteen days. The product had a good, dry flavour and homogeneous consistency.

Example 5 Preparation of Vegetable Juice Containing the Strain LMG P-17806

A vegetable juice was prepared by mixing carrot concentrate and an orange juice concentrate in equal parts so that pH of the finished mixture became pH 3.9. The mixed beverage was heat-treated and added with 1% and 0.1%, respectively, of LMG P-17806 concentrate. The content of LMG p-17806 became 100 and 10 millions per ml, respectively. The beverages were stored at +7°C for four weeks. The flavour of the product was not affected and no influence by storing or addition of LMG P-17806 was observed. Addition of LMG P-17806 to a content exceeding 50 millions, however, seemed to reduce the decrease in vitamin C. In a product without LMG P-17806, like in the product having 10 millions bacteria per ml, the content of vitamin C decreased from 25 mg/100g, before the storage to 18 mg/100g after four weeks. With the addition of 100 millions LMG P-17806 ml the content of vitamin C only decreased to 22 mg/100g, i.e. more than 50% lower decrease in the content of vitamin C. LMG P-17806 survived well and the content of LMG P-17806 after four weeks was only about 40% lower than in fresh

product independent of the amount added.

In another experiment the same fruit beverage was added with 5% LMG P-17806. This gave a tang, which grew worse during storage time.

Example 6 Preparation of Pap Powder

Pap powder is manufactured in the common way. LMG P-17806 concentrate according to the above is lyophilized after mixing with corn starch and stored after being packed in an oxygen-tight wrapping at a temperature of -20°C. The lyophilised preparation contained 61 billions LMG P-17806 per gram. The pap powder was dried-mixed with 0.03% LMG P-17806 and packed in an atmosphere of nitrogen gas in an oxygen tight wrapping. The powder was stored initially for three months at +12°C and thereafter at a room temperature for additional four months. The content of LMG P-17806 is apparent from Table 9 below. The pap powder gave good possibilities for survival to LMG P-17806 and therefore it is possible to prepare, for instance, baby food products having a high content of LMG P-17806.

Table 9

Lyophilized preparation	Powder before addition	Powder after addition	After 3 months	After 7 months
61,000	<0.0001	20	18	6.5

The content in the Table above are given in millions per gram powder.

The pap powder was dissolved in 9 parts of water of +50°C and the content of LMG P-17806 determined. The content was 1 million LMG P-17806 per ml beverage in the case that six months' old powder was used. The pap could not be distinguished from common product. After storing for one night in room temperature, however, the pap added with LMG P-17806

tasted slightly acid and had pH of 5.7.

In a second experiment the effect of the addition of 1% and 0.001%, respectively, of lyophilized LMG P-17806 preparation was examined. The contents thereof in the pap powder after the addition were 570 and 0.8 millions per gram respectively.

The powders were stored in a corresponding way and examined after storage for 5 months. After dissolution as above the content in the prepared pap was 800 millions, respectively for the low addition less than 10,000 per ml. With the high addition a slightly acid flavour was noted already after storing for four hours of the pap at body temperature.

Example 7 Preparation of Dried Powder for Use as "Medical Food"

A lyophilised LMG P-17806 concentrate was mixed with different amounts of corn starch in the proportions in 1:1, 1:9, 1:99 and 1:999, respectively. The mixed powders were stored in small sachets with 1 to 100 g per sachet. The material of the sachets was impervious to oxygen and water vapour. The sachets were stored in freezer, refrigerator and room temperature, respectively. The content was then used as an additive to beverage by mixing the powder with the beverage before the beverage being consumed. The corresponding survival as in example 6 above was obtained. The powder with the proportions 1:1 was also packed in gelatin capsules with 0.4 g per capsule. The number of bacteria per capsule was 10 billions. The capsules were blister-packed in a material with good barrier properties against oxygen and water vapour. The capsules were stored in the same way as stated above and corresponding good survival results were obtained.

Example 8 Preparation and Use of Bacterial Mixes

LMG P-17806 can be mixed with other lactobacilli without inhibiting them. LMG-17806 does not seem to form any substances which are inhibiting other lactobacilli or lactococci where LMG P-17806 are co-cultivated with yoghurt

cultures, sour milk cultures with lactococci or other lactobacilli species such as L. acidophilus, L. fermentum or L. rhamnosus. Experiments with mixtures of lyophilized preparations containing all these bacteria have indicated unchanged storage properties whether mixing was carried out with corn starch or with pap powder. After the solution of the pap powder and a storage for 12 hours at body temperature and 12 hours at room temperature no negative influence by LMG P-17806 could be traced either on the total content of lactobacilli or on the content of either of the bacteria.

In an experiment to produce probiotic sour milk 0.5% of sour milk culture and 0.6% of lyophilized concentrate of each of L. acidophilus NCFB 1748, L. fermentum KLD and LMG p-17806 were added to normal treated product milk. However, only 0.1% KLD was added, because higher additions gave rise to tang and to a bad coagulum. Milk was stored for 19 hours at room temperature. Then the pH thereof was 4.50. The milk was cooled to +10°C, packed and stored at +6°C or up to 14 days. The results are evident from the following table 10.

Table 10

Lactic acid bacteria	Before cooling	After 5 days	After 14 days
Lactococci	730	810	580
L. acidophilus	135	142	131
L. fermentum	12	14	9
LMG P-17806	112	126	109

The contents are in millions per ml of the different bacteria.

80 healthy test subjects were given the products to eat in connection with a conference journey to Istanbul in Turkey. The group was divided into two, one eating a sour milk according to the above with only LMG P-17806 whereas the other ate the probiotic sour milk with three different probiotic

strains. The test subjects were on the conference place for 8 days. They started to eat the products two days before departure to the conference place and continued to eat for four days after home-coming. The products were eaten as 3 snacks evenly spread during the day with 150 g/meal. All test subjects except 2 persons of the sour milk group declared that they ate the product according to the scheme. In an inquiry they were asked to state discomforts from the gastrointestinal tract in the form of stomach pains, tensions, diarrhoea or constipation on a scale of 3 degrees. Apart from diarrhoea there was a difference so far that 5% of the test subjects which only had eaten LMG P-17806 stated that they had had serious or very serious diarrhoea during at least two days whereas this frequency only was 22% in the group which ate the probiotic sour milk.

Thus it seems as if a mix of several different lactobacilli might be still more effective than only one single strain of bacteria. This can be due to the fact that the lactobacilli administered had different properties.

C L A I M S

1. Strain of *Lactobacillus* useful as probiotics in food and naturopathic medicines and which is resistant in-vitro against hydrochloric acid and gastric juice and tolerates bile salts without deconjugating them, whereas a strong assimilation is occurring and which has a good survival at passage through the stomach and the gastrointestinal tract and which strain is growing optimally at about 37°C, **characterised** in

that the strain is *Lactobacillus paracasei* subsp. *paracasei,* which is a Gram-positive, homofermentative, rod-shaped bacterium capable of producing L-lactic acid and

in that it contains three plasmids having a size of 2.2, 4.36 and 9.1 Kb, respectively.

2. Strain according to claim 1, **characterised** in that it contains 44% GC.

3. Strain according to claim 1 or 2, characterised in that it has been isolated from samples from the gastrointestinal micro-flora of humans.

4. Strain according to claim 1, 2 or 3, characterised in that the strain is fermenting ribose, adonitol, galactose, glucose, fructose, mannose, sorbose, mannitol, sorbitol, N-acetyl-glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, D-turanose and D-tagatose.

5. Strain according to claim 1, 2, 3 or 4, **characterised** in that it is provided as a concentrate in the form of frozen or lyophilized powder.

6. Composition of a strain according to any of the preceding claims for use in food and/or as naturopathic medicines, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* is added to a milk product, a fermented milk product, fruit or vegetable beverage such as vegetable juice, citrus fruit juice or a juice of another fruit or vegetable in a content of between 0.001% and 5%, preferably 0.01% and 1%.

7. Composition of a strain according to any of claims 1-4 for use as a probiotic in food and/or naturopathic medicines, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* according to the invention is co-cultivated with yoghurt cultures and sour milk cultures with lactococci or other Lactobacillus species such as *L. acidophilus, L. fermentum* or *L. rhamnosus*.

8. Composition according to claim 6 or 7, **characterised** in that the strain *Lactobacillus paracasei* subsb. *paracasei* according to the invention is present in a content of between 1 million and 10,000 millions living bacteria per gram of composite product.

9. Product containing the strain *Lactobacillus paracasei* subsp. *paracasei* according to any of claims 1-4, **characterised** in that the product consists of

a milk product, a fermented milk product, a vegetable or fruit beverage or pap powder all containing the strain *Lactobacillus paracasei* subsp. *paracasei* in a content of 5 x 10^5 - 5 x 10^9, usually 1 x 10^5 - 10^9 living bacteria, corresponding to 0.0005-0.5% of the product;

or a concentrated naturopathic medicine ("Medical Food") wherein the strain *Lactobacillus paracasei* subsp. *paracasei* is present at a content of 1,000-100,000 millions living bacteria corresponding to 0.001-100% of the product.

10. Product according to claim 8 containing the stated content of bacteria, **characterised** in

that in case of food products it further contains a small proportion, e.g. 0.001-0.1%, of yeast extract or other substances which contribute to growth or survival of *Lactobacillus paracasei* subsp. *paracasei* in the product,

and that it in case of naturopathic medicines it contains substances of importance for survival of the bacteria and/or residues of the culture substrate.

11. Product according to claim 9 or 10, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* is

provided as a concentrate in frozen or lyophilized condition for mixing in direct connection with consumption occasion into a milk product, a fermented milk product, a vegetable or fruit beverage, in pap powder or in a concentrated naturopathic medicine (so-called "Medical Food").

12. Product containing a strain of *Lactobacillus paracasei* subsp. *paracasei* according to any of claims 1-5, **characterised** in that it is used

for children for the purpose of alleviating atopic problems;

for elderly persons in order to correct altered micro-flora caused by normal changes by age or an altered secretion of hydrochloric acid;

and for persons in general in order to normalise the intestinal flora in which case the content of clostridia bacteria is decreasing, bifidobacteria is increasing and high contents of coliformic bacteria are decreasing.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/02263

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 1/20 // (C12N 1/20, C12R 1:225)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 13333687, Biosis no. 99333687, Mercenier A et al: "Development of lactic acid bacteria as live vectors for oral or local vaccines"; & Advances in Food Sciences 18 (3-4). 1996. 73-77 ---	1-12
X	Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 11493893, Biosis no. 98093893, Harty D W S et al: "Pathogenic potential of lactobacilli"; International Journal of Food Microbiology 24 (1-2). 1994. 179-189 ---	1-12

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 1999	21 -03- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 98/02263

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9709448 A1 (OULUTECH OY), 13 March 1997 (13.03.97), see page 3, line 32 - page 4, line 2, page 7, lines 25-34	1-12
A	Chemical Abstracts, Volume 125, No 23, 2 December 1996 (02.12.96), (Columbus, Ohio, USA), Klein Guenter et al, "Total soluble cytoplasmatic protein patterns of Lactobacillus rhamnosus and Lactobacillus paracasei from different habitats", page 557, THE ABSTRACT No 296749m, Mikrooekol. Ther. 1995, 23, 179-187	1-12
A	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 11400418, Biosis no. 199800181750, Savova T et al: "Lactobacillus casei: Survival in the gastrointestinal tract and biostimulating activity"; & Zhivotnov"dni Nauki 33 (7-8):p55-57 1996	1-12
A	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 02137076, Biosis no. 000063052076, Gilliland S E et al: "De conjugation of bile acids by intestinal lactobacilli"; & Appl Environ Microbiol 33 (1). 1977 15-18	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/99

International application No.
PCT/SE 98/02263

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9709448 A1	13/03/97	AU 6877396 A	27/03/97
		FI 102298 B	00/00/00
		FI 954194 A	08/03/97